Epidermal Dysplasia and Abnormal Hair Follicles in Transgenic Mice Overexpressing Homeobox Gene *MSX-2*

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The homeobox gene *Msx-2* is expressed specifically in sites of skin appendage formation. To explore its part in skin morphogenesis, we produced transgenic mice expressing *Msx-2* under the control of the cytomegalovirus promoter. The skin of these transgenic mice was flaky, exhibiting desquamation and shorter hairs. Histologic analysis showed thickened epidermis with hyperproliferation, which was restricted to the basal layer. Hyperkeratosis was also evident. A wide zone of suprabasal cells were misaligned and coexpressed keratins 14 and 10. There was reduced expression of integrin β1 and DCC in the basal layer. Hair follicles were misaligned with a shrunken matrix region. The dermis showed increased cellularity and empty vacuoles. We suggest that *Msx-2* is involved in the growth control of skin and skin appendages. *Key words: adhesion molecules/integrin/keratin/skin. J Invest Dermatol 113:230–237, 1999*

Vertebrate skin consists of multiple layers which interact via growth factors to regulate morphogenetic processes. Molecular mechanisms underlying the specification of cells in the developing skin are still not well understood. During the formation of stratified epithelia, cell proliferation is limited to the basal layer, which contains stem cells and transient amplifying cells. These cells express integrin (Jones *et al*, 1995) and keratin (K) 5 and K14 (Fuchs and Green, 1980). The generation of the suprabasal layer is accompanied by the loss of integrin, expression of K1, K10, and other mature keratinocyte markers such as filaggrin and involucrin (Fuchs and Byrne, 1994). Stratification of the skin involves complex regulation involving calcium, the protein kinase C pathway, jun, fos, ras, and P21 (Missero *et al*, 1996; Rutberg *et al*, 1996). Proliferation of the basal cells was recently shown to be regulated by the sonic hedgehog/patched pathway as de-regulation of this pathway leads to carcinoma (Johnson *et al*, 1996; Dahmane *et al*, 1997; Oro *et al*, 1997). Despite such progress, much remains to be learned about how the growth of epidermis and skin appendages is regulated.

We are interested in the molecular events underlying the temporal and spatial control of this process. We have used skin appendages as a model to explore the function of these regulatory molecules (Chuong *et al*, 1996). Among the molecules is Msx-2, a homeodomain-containing protein that is expressed in a variety of sites during organogenesis, particularly in sites where tissue interactions take place (Davidson, 1995). In craniofacial development, a mutation of the human *Msx-2* gene was found to correlate with premature closure of the cranial sutures, a condition found in individuals with Boston type craniosynostosis (Jabs *et al*, 1993). This condition was mimicked in transgenic mice expressing a mutant or normal mouse *Msx-2* gene (Liu *et al*, 1995). Transgenic mice with a targeted disruption of the *Msx-1* gene also exhibit abnormalities in the craniofacial bones (Satokata and Maas, 1994) demonstrating the functional involvement of *Msx* genes in the growth control of calvaria. In the limb bud, Msx-2 is expressed in the apical ectodermal ridge and both Msx-1 and Msx-2 are expressed in the progress zone mesoderm subjacent to the apical ectodermal ridge (Yokouchi *et al*, 1991; Sumoy *et al*, 1995). Msx expression is apical ectodermal ridge dependent and fibroblast growth factor dependent and is proposed to be important for the function of the progress zone (Ferrari *et al*, 1998; Muneoka and Sassoon, 1992). In the mammary gland, Msx expression is involved in epithelial–mesenchymal interactions that regulate gland development and Msx-2 expression was found to be regulated by estrogen levels (Friedmann and Daniel, 1996; Phippard *et al*, 1996). *In vitro* studies further substantiate the role of *Msx* genes in growth control. Msx-1 modulates myogenic cell differentiation/proliferation by promoting proliferation (Wang and Sassoon, 1995). Recently, a study was conducted to search for induced genes that can reverse the v-Ki-ras transformed NIH 3T3 cell phenotypes. Interestingly, a carboxy-terminal fragment of Msx-2 was isolated. Expression of endogenous Msx-2 is also upregulated in NIH3T3 cells transfected with v-Ki-ras (Takahashi *et al*, 1996). These data suggest that *Msx* genes play a key part in growth control.

Msx-1 and *Msx-2* are found to be expressed in developing skin in the feather placode (Noveen *et al*, 1995), mouse hair follicle epithelia (Reginelli *et al*, 1995), and human skin epithelia (Stelnicki *et al*, 1997). Their roles in skin and skin appendage development, however, have not been defined. In *Xenopus*, BMP-2 can enhance the formation of epidermis in an Msx-1-dependent pathway (Suzuki *et al*, 1997). *Msx-1* knockout mice have abnormal tooth and hair growth (Satokata and Maas, 1994). LEF-1 was shown to be involved in the induction of epithelial appendages (Zhou *et al*, 1995), and

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Abbreviations: BMP, bone morphogenetic protein; CMV, cytomegalovirus; DCC, deleted in colorectal carcinoma; LEF, lymphocyte enhancer-binding factor; Msx, muscle segmentation homeobox homolog; NCAM, neural cell adhesion molecule.

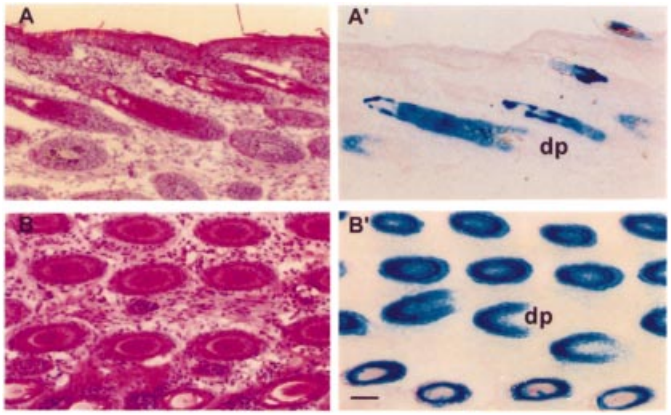

Figure 1. Endogenous Msx-2 expression as shown in transgenic mice with a Msx-2 reporter driving expression of LacZ. Neighboring transverse section (*A*, *A′*) and cross-section (*B*, *B′*) of skin from transgenic mice expressing LacZ from the full length *Msx*-2 promoter were stained with hematoxylin and eosin (*A*, *B*) or for β-galactosidase (*A′*, *B′*). ep, epidermis; dp, dermal papilla. *Scale bar*: 60 μm.

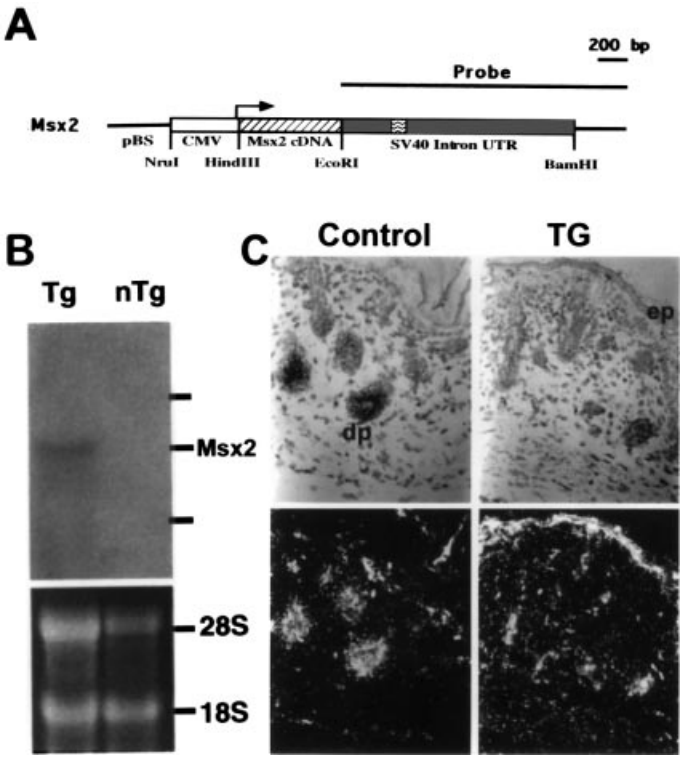

Figure 2. Production of transgenic mice overexpressing Msx-2. (*A*) The plasmid construct of the mutated (Pro7His) *Msx*-2 driven by the CMV promoter is shown. Regions representing the probes specific to the SV40 intron are also shown. (*B*) Total RNA from the skin of CMV-Msx-2 transgenic (Tg) and nontransgenic (nTg) mice were extracted, run on an agarose gel, and blotted with a probe specific to SV40 (*A*). A band of about 4 kb is seen which is the size of the construct. The *bottom panel* is the control showing the expression of 18S and 28S rRNA. (*C*) *Msx*-2 mRNA *in situ* hybridization in control (*A*, *B*) and transgenic mice (*A′*, *B′*). Cross-sections of skin were viewed with bright-field (*A*, *A′*) and dark-field (*B*, *B′*) optics. *Msx*-2 is present in the follicles including the matrix region in both sibling and transgenic mice. Note that the follicles in the transgenic mice are a smaller size and irregularly shaped in comparison with the nontransgenic controls. See **Figs 4** and **5** for hematoxylin and eosin staining. *Msx*-2 overexpressing mice express large amount of *Msx*-2 in the epidermis and low and diffuse staining in the dermal mesenchyme. ep, epidermis; dp, dermal papilla.

its molecular pathway involves Msx (Kratochwil *et al*, 1996). These results strongly support the importance of the Msx pathway in skin development.

In this study we provide further insight into the role of Msx-2 in skin development. We document abnormal skin phenotypes in transgenic mice in which *Msx-2* is overexpressed under the control of a cytomegalovirus (CMV) promoter. These mice were produced before and used to study the role of Msx-2 in craniofacial development (Liu *et al*, 1995). Overexpression of Msx-2 in skin *in vivo* causes epidermal dysplasia and abnormal hair growth. The spatially and temporally ordered process of epidermal growth control is disrupted. These data suggest that Msx-2 is intimately related to skin morphogenesis, and is an important element in the pathways that modulate the timing and locale of cell proliferation and differentiation.

MATERIALS AND METHODS

Msx-2 transgene constructs and the making of transgenic mice The construction and generation of the *Msx-2* promoter-lacZ reporter mice were described before (Liu *et al*, 1994). Briefly, this transgenic mouse contains a construct placing the regulation of the lacZ gene under the transcriptional control of the 5.2 kb *Msx-2* promoter. Expression from this construct is consistent with the normal expression pattern of *Msx-2* in skull and limb (Liu *et al*, 1994); however, the skin expression has not been examined and is shown here.

Transgenic mice with constitutively active *Msx-2* expression were reported before and phenotypes in calvaria fusion discussed (Liu *et al*, 1995). Skin phenotypes are reported in this study. The plasmid was constructed by taking a fragment of the immediate early promoter of the human CMV from pRc/CMV (Invitrogen, Carlsbad, CA) as a *Sal*I/*Hin*dIII fragment and ligating it to compatible sites on pBluescript SKII (Liu *et al*, 1995; Stratagene, La Jolla, CA). A 2.1 kb *Bam*HI/*Pst*I fragment from pKSV10 (Pharmacia, Milwaukee, WI) was then inserted into the *Bam*HI site to supply a polyadenylation signal and an exogenous intron. A 1.0 kb fragment of the mouse *Msx-2* cDNA was inserted into the *Hin*dIII/*Eco*RI sites of this expression plasmid. The 3.7 kb transgene fragment was freed from vector sequences by digesting with *Nru*I and *Bam*HI, and was gel purified and microinjected into mouse zygotes derived from B6CBA F1 donors.

Transgenic mice were generated and analyzed as described (Hogan *et al*, 1986). Briefly, 4 wk old B6CBA F_1 female mice were superovulated and mated with adult B6CBA F_1 males. Prior to microinjection, isolated fertilized oocytes were cultured in M16 medium at 37°C in a humidified incubator supplemented with 5% CO_2. Approximately 2 pl of linearized DNA (1 μg per ml) were microinjected into the male pronucleus of each oocyte. Surviving oocytes were transferred to the oviducts of 8 wk old pseudopregnant CD1 females. At 2 wk of age, live born animals were analyzed for the presence of the transgene by southern blot hybridization. Three founder lines were used in this study. Mice were housed in a pathogen-free environment in filter-top cages. To maintain pathogen-free conditions, cages, bedding, and food were sterilized. In addition, bedding was changed twice a week in a laminar flow hood. Health surveillance was performed once a month.

β-galactosidase expression analysis Mouse skin was fixed in 4% paraformaldehyde for 30 min and was then embedded in OCT medium (Miles, Elkhart, IN) for frozen sections. Sections were incubated overnight at room temperature in X-Gal staining solution (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 0.1% 4-chloro-5-bromo-3-indolyl β-galactosidase (X-gal, Research Organics, Cleveland, OH), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide in phosphate-buffered saline).

Histologic, immunologic, and *in situ* hybridization analyses For immunoalkaline phosphatase staining, the tissues were fixed with Bouin's fixative, dehydrated through an alcohol series, and embedded in paraffin. Sections were rehydrated before staining and blocked for 30 min at room temperature with Zeller's solution. The solution was then replaced with 1 × 0.05% Tris buffered saline with Tween–20 (TBST) solution containing 1:300 dilution of antibodies. After overnight incubation, sections were washed three times in TBST and then treated with alkaline phosphate conjugated secondary antibody diluted in TBST for 2 h. The slides were washed in TBST and placed in alkaline phosphate buffer for 5′, and immunostaining was detected with nitroblue tetrazolium and 5-bromo-4-chloro-3 indolyl phosphate (Promega, Madison, WI) substrates (Jiang *et al*, 1998). Antibodies sources: K14, Dr Fuchs (Stoler *et al*, 1988); K10, Dr Yuspa (Roop *et al*, 1987); integrin β1 (Chemicon, Temecula, CA); DCC

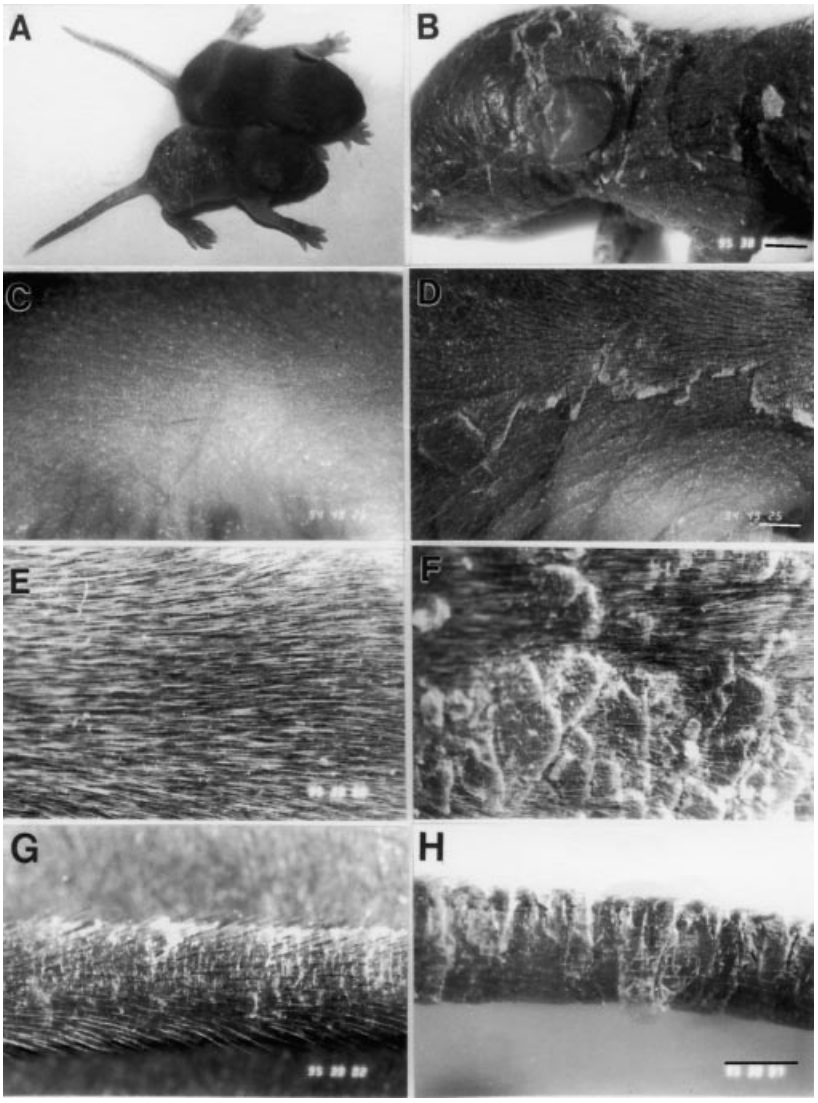

Figure 3. Abnormal skin in the Msx-2 overexpressing transgenic mice. In panel A, the sibling is on the top and the transgenic mouse is on the bottom. (*C*, *E*, *G*) Sibling. (*B*, *D*, *F*, *H*) Transgenic mice. Postnatal day 5. (*A*) Mice from the same litter. In transgenic mice, the occipital, posterior body, and flank regions are usually affected most. (*B*) Anterior body of the transgenic mouse. It shows scaliness and desquamation in the skin, particularly in the occipital region. (*C*, *D*) Flank region near the abdomen. The skins are flaky and peeling off in the transgenic mice. (*E*, *F*) Posterior dorsal skin over the trunk. Note the thickened and scaly appearance in the transgenic mice. Hair growth is inhibited in this region. (*G*, *H*) Tail. Transgenic mice show severe hyperkeratotic changes, scaliness, and shorter hair. *Scale bars*: *B*, *G*, *H*, 1 mm; *C–F*, 500 μm.

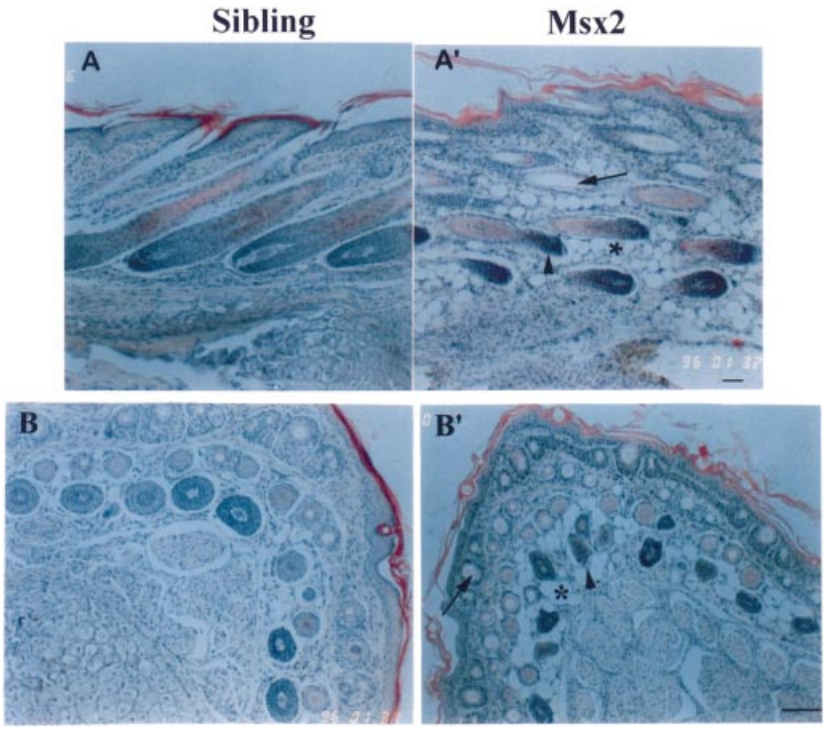

Figure 4. Histologic sections of skin from normal and transgenic mice. Paraffin sections of postnatal day 5 sibling (*A*, *B*) and transgenic (*A′*, *B′*) mouse tails shown in longitudinal sections (*A*, *A′*) and cross-sections (*B*, *B′*). Hematoxylin and eosin staining. Note the thickened epidermis and some irregular epidermal invaginations with embedded hair follicles. There are shrunken hair matrixes (*arrowheads*), although the follicle became bigger distally. There are also increased empty hair follicles (*arrows*). In the dermis, there are empty vacuoles (*). *Scale bars*: *A*, 100 μm; *B*, 200 μm.

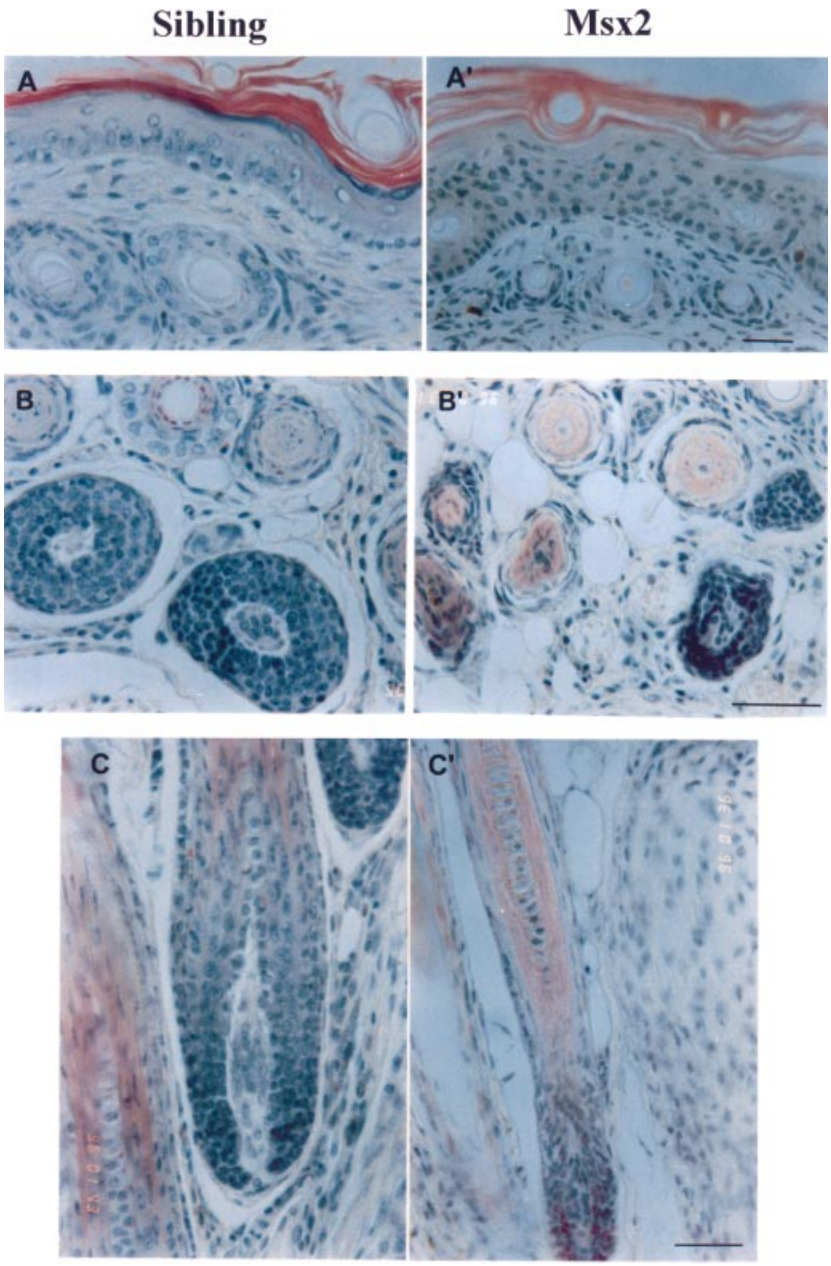

Figure 5. Epidermal dysplasia and abnormal hair follicles in Msx-2 transgenic mice. (*A*, *A′*) Epidermis. The P5 sibling epidermis show a well organized basal layer with suprabasal layer and stratum corneum laid in order. Transgenic mice showed a thickened suprabasal layer with increased cellularity that is misaligned. There is also increased cellularity in the upper dermis. There are no inflammatory cells. (*B*, *B′*) Hair follicles, cross-sections. Note the shrunken matrix regions resulting in irregular follicle contour in the transgenic mouse. (*C*, *C′*) Hair follicles, longitudinal sections. Note the bulb regions of the transgenic mice are poorly developed and there are fewer matrix epithelial cells and thinner outer root sheath. *Scale bar*: 100 μm.

('deleted in colorectal cancer') (Chuong *et al*, 1994); and neural cell adhesion molecule (NCAM) (Chuong *et al*, 1982).

For *in situ* hybridization, skin on the dorsal side of the body was fixed in freshly prepared 4% paraformaldehyde, dehydrated, embedded in paraffin, and sectioned. Sections (7 mm thick) were mounted on 3-triethoxysilylpropylamine (Sigma, St Louis, MO) treated slides. Sections were deparaffinized, hydrated, treated with 10 mg per ml proteinase K, fixed, and acetylated. The anti-sense *Msx-2* RNA probe was transcribed off pCRIIcMsx-2 by using SP6 RNA polymerase (Pharmacia) and the sense probe was transcribed using T7 RNA polymerase in the presence of ^{35}S uracil triphosphate (Amersham, Arlington Heights, IL). Probes were not hydrolyzed. Hybridization was carried out overnight at 50°C at a probe concentration of 50 000 cpm per ml. Slides were washed twice in 4 × sodium citrate/chloride buffer at 50°C and once at 65°C in 50% formamide, 2 × sodium citrate/chloride buffer, 10 mM dithiothreitol. Unbound probes were then degraded by treating slides with 20 mg per ml RNase A at 37°C for 30 min. A final stringent wash was done at 50°C in 0.1 × sodium citrate/chloride buffer. After dehydration, slides were coated with photographic emulsion (Amersham) and were exposed for 14 d at 40°C. Slides were developed using Kodak D-19 developer and fixer.

Bromodeoxyuridine (BrdU) labeling BrdU (50 μg per kg body weight) was injected to the mouse 1 h prior to being killed. The mouse skin was then fixed with 4% paraformaldehyde, dehydrated, and embedded in paraffin. After sectioning, the slides were de-waxed, the cellular DNA was denatured in 1.5 M HCl for 30 min and neutralized with phosphate-buffered saline for 1 h. Proliferating cells were stained with monoclonal anti-BrdU antibody *following* the recommended protocol (Boehringer Mannheim, Indianapolis, IN).

RESULTS

Endogenous *Msx-2* expression pattern The normal expression pattern of *Msx-2* has been shown to be in the matrix epithelial cells of hair follicles and the nail bed by *in situ* hybridization (Reginelli *et al*, 1995). We have used *in situ* hybridization and a *Msx-2* promoter driven reporter to examine endogenous *Msx-2* expression. The promoter-reporter method is used in conjunction with *in situ* hybridization because it is more sensitive and allows future analysis of the regulation of *Msx-2* expression. Transgenic mice with β-galactosidase driven from the full 5.2 kb length *Msx-2* promoter were produced (Liu *et al*, 1994). Neighboring transverse and cross-sections of the skin of these mice at day 7 were stained for hematoxylin and eosin (**Fig 1*A*, *B***) and for β-galactosidase (**Fig 1*A′*, *B′***). The β-galactosidase expression pattern shows that

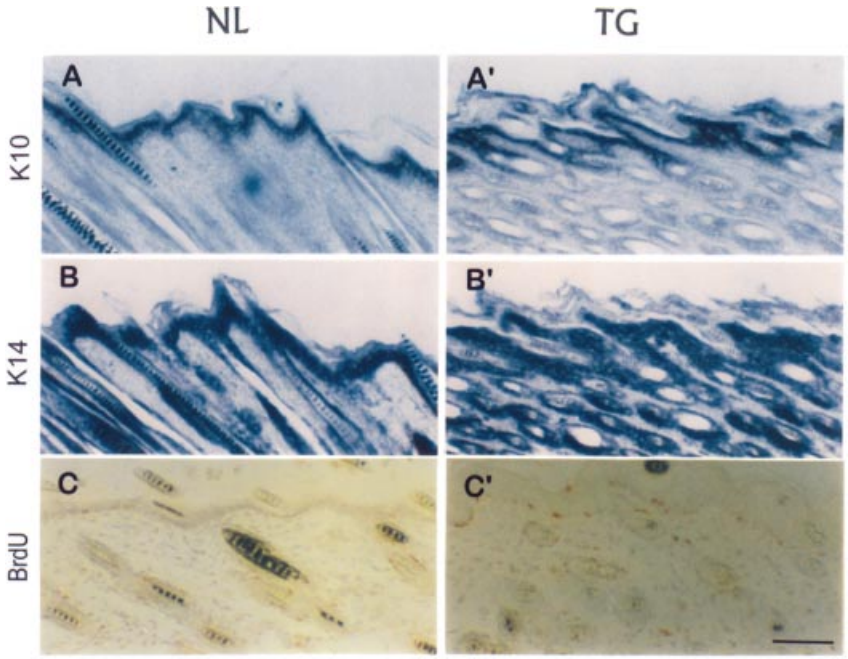

Figure 6. K10, K14, and BrdU labeling in Msx-2 transgenic mice. Paraffin sections of sibling (*A–C*) and transgenic (*A′–C′*) mice were stained with antibodies to K10 (*A*, *A′*) and K14 (*B*, *B′*). In the normal mice, K10 is in the suprabasal and K14 in the basal layer and at most one more layer above the basal layer. In the transgenic mice, a major portion of the thickened epidermis is positive for both K10 and K14. (*C*, *C′*) BrdU labeling. Note BrdU labeling in the skin of transgenic mice is still limited to the basal layer, but the number is higher. *Scale bar*: 100 μm.

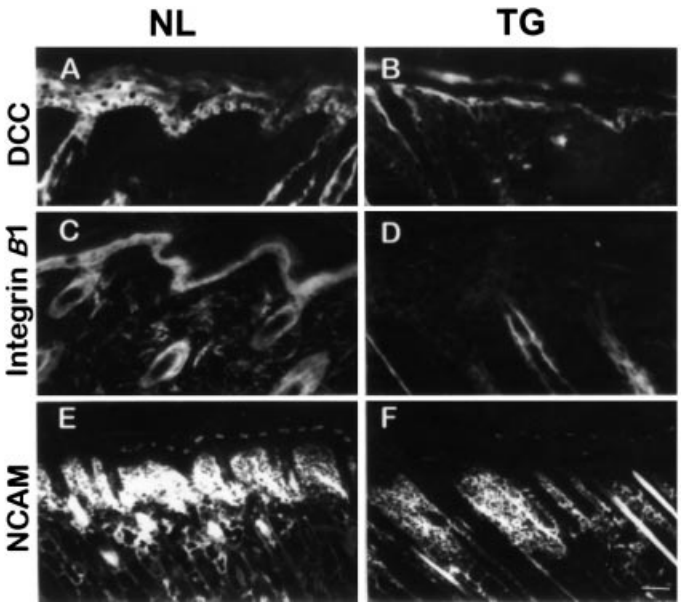

Figure 7. Expression of integrin β1, DCC, and NCAM in Msx-2 transgenic mice. Frozen sections of sibling (*A*, *C*, *E*) and transgenic (*B*, *D*, *F*) mice were stained with antibodies to DCC (*A*, *B*), integrin β1 (*C*, *D*), and NCAM (*E*, *F*). Note DCC and integrin β1 are expressed in the basal layer of the normal epidermis, but becomes very weak in the epidermis of the transgenic mice. NCAM is in the superficial dermis of the control mice. In the CMV-Msx-2 mice, the regions of superficial dermis are expanded and the zone expressing NCAM also becomes wider. *Scale bar*: 100 μm.

Msx-2 is expressed in the hair follicles including the matrix. *In situ* hybridization showed a similar pattern (**Fig 3*A*, *A′***).

Abnormal skin in *Msx-2* overexpressing transgenic mice To produce transgenic mice overexpressing *Msx-2*, *Msx-2* was placed under the control of the CMV immediate early gene promoter (**Fig 2*A***). Transgenic expression was verified using northern blot hybridization with probes to the SV40 sequences. A 3.1 kb band representing transgene expression can be detected in RNA extracted from the skin of transgenic but not from nontransgenic mice (**Fig 2*B***). The overexpression of *Msx-2* was further verified by *in situ* hybridization (**Fig 2*C***). The distribution in control embryos was similar to the control (Reginelli *et al*, 1995). In the transgenic mice, *Msx-2* was highly expressed in the epidermis. *Msx-2* was also in the hair follicles and the distorted pattern was due to the misshapen and shrunken hair follicles (see below, **Figs 4** and **5**). A lower and diffuse presence of *Msx-2* was also seen in the dermal mesenchyme.

Three independent mouse lines were generated and all showed similar skin phenotypes. Newborn transgenic mice showed marked skin abnormalities. The major gross changes were thickened skin with hyperkeratosis, desquamation and flakiness (**Fig 3**). In some regions fewer hairs were observed. This may be a failure of the hair shaft to emerge out of the skin due to inappropriate hair follicle angles (see below). The severity of changes was regional. The most severe region was the tail, then the dorsal posterior skin, flank region, occipital region, and then other trunk regions. All regions with these abnormal changes gradually decreased in size over time, becoming patches of abnormality. These alterations were observed in both male and female transgenic mice. The phenotypes were more clear in the first 2 wk after birth. In transgenic mice older than 1 mo, the skin phenotypes were no longer apparent.

Histologic sections showed abnormal histology in the epidermis, dermis, and hair follicles (**Figs 4** and **5**). In the epidermis, the stratum corneum was thicker and accompanied by hyperkeratotic changes; therefore, more hair follicles were embedded in the epidermis. Hair follicles were misaligned. The follicles spread through different depths of the dermal layer. The angles were irregular and some hair shafts appeared to fail to find a way out. The number of empty hair canals was higher in transgenic mice than in control siblings. In the dermis, there were vacuolated tissues that replaced normal connective tissue (**Figs 4** and **5**).

At a high power view, there was increased cellularity and dysplastic changes in the epidermis of the transgenic mice. Cells in the stratum basale still showed the palisade arrangement, but the cell density was higher than in the sibling. In the suprabasal layers, the orderly and parallel transition of cells from the stratum spinosum to the corneum in the normal mice was disrupted. Instead, many cells were randomly distributed in the suprabasal layers of transgenic mouse skin (**Fig 5*A*, *A′***). In the superficial dermis, the regions immediately adjacent to the epidermis showed hypercellularity in the transgenic mice (**Fig 5*A*, *A′***). Overall, the cell nuclei in the transgenic mice were dense and tightly packed, unlike the normal nuclei that were usually plump with distinct nucleoli. No inflammatory cells were observed.

In the hair of nontransgenic mice, *Msx*-2 was expressed in the matrix epithelium surrounding the dermal papilla. Increased doses of Msx-2 produced cells with condensed and elongated nuclei, rather than the round and plump morphology found in the sibling (**Fig 5*B–C′***). The matrix region was narrower than the control, and keratinization started earlier (more proximal) than in the control, making the matrix regions of the transgenic mice shorter than the sibling. Overall, there were fewer matrix epithelial cells in the transgenic mice. Regions corresponding to the inner root sheath were also poorly developed. Interestingly, more distal to this region, the cortex and medulla regions appeared close to normal. Therefore, there was a poorly developed bulb region and hair follicle (**Fig 5*C, C′***). At the proximal level of the follicle, the concentric rings of the outer root sheath, inner root sheath, and matrix cells in the normal hair (**Fig 5*B, B′***) were lost and replaced by irregularly arranged cells. More distal, however, the cortex and medulla appeared to be more normal.

Disrupted growth control in the skin of *Msx-2* transgenic mice To test the keratin type expressed by epidermis of the CMV-Msx-2 transgenic mice, we examined the expression of K10 and K14. K10 is normally expressed in the suprabasal keratinocytes (Fuchs and Green, 1980) (**Fig 6*A***). In *Msx*-2 overexpressing transgenic mice, the thickened layer of suprabasal keratinocytes expressed K10, but the basal layer remained K10 negative (**Fig 6*A′***). In control mice, K14 was expressed in the basal layer and in part of the outer root sheath of hair follicles (**Fig 6*B***). In *Msx*-2 transgenic mice, K14 expression extended beyond the basal layer and was positive in most of the suprabasal keratinocytes (**Fig 6*B′***). The most superficial layer, representing more differentiated keratinocytes, however, remained negative for K14. Thus the majority of the dysplastic keratinocyte population that constitutes the thickened epithelium displayed an ambiguous property by expressing both basal and suprabasal keratins.

We then tested for cell proliferation. BrdU was used to label *Msx*-2 transgenic and control mice for 1 h before the animals were killed. In the basal layer, BrdU-labeled cells were mildly increased. There were no labeled nuclei in the suprabasal layer, however, and all proliferating cells were still limited to the basal layer (**Fig 6*C, C′***), suggesting that overexpression of *Msx-2* in the epidermis did not cause an overwhelming proliferation of the epidermal cells. The results suggest that *Msx-2* may be involved in regulating the timing of proliferation and differentiation, namely growth control. Ectopic expression of *Msx*-2 caused loss of regulated control, probably by accelerating the whole process.

To test the effect of *Msx* expression on cell interactions, we examined the expression of cell surface molecules implicated in growth control. We showed recently that DCC is predominantly expressed in the basal layer of stratified epithelium (Chuong *et al*, 1994; Combates *et al*, 1997) (**Fig 7(*A***). In *Msx*-2 transgenic mice, DCC expression became weak and diffuse in both the basal and suprabasal layers, whereas some staining remained in the outer root sheath (**Fig 7*B***). Normally, integrin β1 is expressed in the basal layer and disappears in the suprabasal layer when cells start to differentiate (Jones *et al*, 1995) (**Fig 7*C***). In *Msx*-2 transgenic mice, integrin β1 was diminished in the basal layer (**Fig 7*D***). Integrin β1 did not disappear in all the tissues in transgenic mice as some integrin β1 staining remained in the outer root sheath of hair follicles. This suggests that the basal cells in CMV-Msx-2 mice have altered cell surface properties and cell interactions may not function properly because some signaling pathways may have been altered.

In normal mice, NCAM was also positive in the superficial dermis, which is composed of dense dermal cells immediately beneath the epidermis and adjacent to the hair follicles (**Fig 7*E***). In *Msx*-2 transgenic mice, this zone of superficial dermis was thickened and remained NCAM positive (**Fig 7*F***). Thus there was increased NCAM expression in the dermis, which may lead to abnormal morphogenetic activity and the observed abnormal architecture of connective tissues, including the many vacuoles.

DISCUSSION

During development, through cell and tissue interactions, epithelial cells differentiate into multiple types of keratinocytes that form skin and skin appendages. It is generally agreed that epithelial stem cells give rise to transiently amplifying cells in the epidermal basal layer and the hair follicle matrix region, from which differentiated keratinocytes are generated in a precise temporal and spatial sequence (Cotsarelis *et al*, 1990; Carroll *et al*, 1995). This progression from proliferation to differentiation must require cell interactions and molecular controls. The mechanisms of this process remain mostly unknown.

The expression patterns of *Msx*-2 in the feather placode (Noveen *et al*, 1995) and hair matrix (Reginelli *et al*, 1995) suggest that Msx-2 is transiently expressed in skin appendage keratinocyte precursors at the time when cells proliferate and acquire competence (the ability to differentiate when receiving appropriate signals). The acquisition of competence is a transient state that requires intricate regulation to keep growth and differentiation in balance. Here we examined the role of Msx-2 in skin and skin appendage development using Msx-2 overexpressing transgenic mice. We reported the phenotypes of hyperkeratosis, desquamation, and flakiness in the skin. The observed phenotypes were regional specific, being more severe in the neck, flank, and tail regions. The phenotypes were also temporal dependent, being more severe in the first 2 wk after birth and gradually recovering. The skin of transgenic mice older than 1 mo appear to be normal. Our preliminary data, however, showed that re-initiation of the hair cycle by wax stripping can cause a renewed difference between control and transgenic mice, which again becomes less obvious 2 wk after the stripping (not shown). The involvement of Msx in the hair cycle is itself interesting, and is currently undergoing investigation. The alleviated phenotypes during aging could be due to transient action of Msx-2 in a developmental stage specific manner, the compensation by other Msx-related pathways, or the silencing of CMV promoters (Loser *et al*, 1998).

Overexpression of *Msx-2* disrupts the molecular environment and the balance of growth control in the skin In normal skin epidermis, cells proliferate only in the basal layer. With appropriate signals, cells begin to differentiate and move upward to form the suprabasal layer and ultimately to cornify. In addition to the distinct cytoarchitecture, characteristic differentiation molecules mark different layers. K5 and K14 are basal keratins expressed only in the basal layer, although the proteins can still be detected beyond the basal layer. K1 and K10 are suprabasal keratins (Fuchs and Byrne, 1994). When Msx-2 was constitutively expressed, the skin became hyperkeratotic and flaky. Histologic examination showed thickened epidermis with disrupted cytoarchitecture. The palisade arrangement of the basal layer was lost, and the parallel stack of thick to thin suprabasal cells was replaced by randomly oriented polygonal cells, suggesting a dysplastic change. We used markers for cell proliferation and cell differentiation to examine the effects of Msx-2 overexpression. BrdU labeling showed that cell proliferation in the Msx-2 overexpressing mice was still limited to the basal layer, although a higher frequency of BrdU labeling was observed. In the suprabasal layer, cells started to express K10 and filaggrin earlier than controls, leading to a thickened layer of keratinocytes with an ambiguous identity characterized by coexpression of both K10 and K14 keratins, markers of undifferentiated and differentiated keratinocytes, respectively. This suggests that the ectopic expression of Msx-2 in stratified epidermis disrupted the balanced proliferation/maturation process of basal cells. Suprabasal keratinocytes differentiate precociously in conjunction with increased cell proliferation in the basal layer.

In the hair, Msx-2 is normally expressed in the matrix (Reginelli *et al*, 1995). This is the region of active cell proliferation that produces the majority of cells for the hair filament. Mice overexpressing Msx-2 had a shrunken matrix region with a reduced number of hair keratinocyte precursors, whereas the morphology

and diameter of the hair shafts, once formed, appeared to be normal. Again misexpression of Msx-2 may alter the flux of cells between proliferation and differentiation states, thus leading to the abnormal hair follicle formation.

What events lie downstream to Msx-2? Cell adhesion molecules such as integrin have been shown to play critical roles in regulating the differentiation of keratinocyte precursors (Bagutti *et al*, 1996). Integrin β1 is expressed in the basal layer and becomes lost when basal cells start to differentiate (Jones and Watt, 1993). Perturbation of integrin–fibronectin interactions using anti-fibronectin antibodies induces precocious epidermal differentiation (Adams and Watt, 1989). Here we observed a suppression of integrin β1 in the basal layer (**Fig 7**). It is possible that the precocious effect of overexpressing Msx-2 in the epidermis may be mediated through suppressed activity of an integrin–extracellular matrix pathway. Another altered adhesion molecule is DCC, a calcium-independent adhesion molecule and a putative tumor suppressor in colon carcinoma (Fearon *et al*, 1990). DCC is highly expressed in the basal layer of epidermis, hair bulge epithelia, and the crypt region of intestinal microvilli (Chuong *et al*, 1994; Combates *et al*, 1997), suggesting a role in regulating cell proliferation. In Msx-2 overexpressing mice, DCC in the basal layer is suppressed (**Fig 7**). The suppression of DCC in the transgenic mice again suggests that the basal cells do not have normal properties and loss of DCC mediated interactions may account for de-regulation of growth control. Change of adhesion molecular profiles in the basal cells of Msx-2 overexpressing transgenic mice may lead them to respond differently to environmental cues. Further studies are required to elucidate the mechanism by which Msx-2 can influence epidermal stratification.

Msx-2 may be involved in regulating epidermis thickness The function of Msx-1 and Msx-2 in development has been studied in osteoblast differentiation (Hoffmann *et al*, 1994), limb bud development (Coelho *et al*, 1993), muscle differentiation (Wang and Sassoon, 1995), and tooth development (Chen *et al*, 1996). Whereas *Msx* genes are expressed in sites of epithelial–mesenchymal interactions, their expression is more sensitive to modulation at the time of growth control. Their exact roles seem to differ depending on the context. Overall, *Msx* genes appear to act as modifiers of transcriptional activity and gene expression (Catron *et al*, 1995) probably through interactions with other molecules such as dlx (Zhang *et al*, 1997) or miz-1 (Wu *et al*, 1997). Msx-1 may also mediate the effect of BMP4 in the formation of the epidermis (Suzuki *et al*, 1997).

In the formation of stratified epithelium, the epidermis can be divided into differentiation and proliferation layers. The border between these two layers is set by the last proliferation event and the initiation of differentiation. We propose a hypothetical gate regulating the flow of cells from a proliferating compartment to a differentiated compartment. Positive and negative regulators would act to keep an appropriate level of transiently amplifying cells proliferating whereas allowing a certain number of cells to progress to differentiation. When this 'gate' is wide open, as may occur in *Msx*-2 transgenic mice, there is a rapid transit into differentiated status. The result is precocious differentiation and accelerated depletion of the transiently amplifying cell pool. This depletion may induce the accelerated proliferation in the basal layer to replenish the cells. The result is a thickened epithelium with hyperkeratosis.

The whole picture of the regulation of epidermal thickness, however, is likely to be much more complex involving the interaction of many cytokines and intracellular regulators (Blessing *et al*, 1994; Missero *et al*, 1996; Rutberg *et al*, 1996). Nevertheless, this study points toward the functional involvement of Msx-2 in mammalian skin development and, is consistent with a role for Msx-2 in regulating the traffic between proliferation and differentiation. Future research will focus on elucidating how the Msx-2 pathway interacts with other pathways in regulating the growth control of keratinocyte precursors, and how these pathways are involved in the physiologic and pathologic conditions involving epithelial development.

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